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In vitro anticancer potential of *Anaphyllum wightii* Schott. against Dalton's lymphoma ascites cell lines and molecular docking studies of β-sitosterol

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Cancer is the second most life threatening noncommunicable disease in humans that challenges the mankind with its multifactorial etiology. Even though various treatment strategies such as radiation therapy, chemotherapy, etc. are commonly used for this disease, researches are being focussed on developing plant-derived novel anticancer compounds that lack side effects. There were no previous reports on the anticancer potential of the ethnomedicinally important plant *Anaphyllum wightii* Schott. (Araceae). Hence, in the present study, we evaluated the *in vitro* cytotoxic effects of *A. wightii* on Dalton's lymphoma ascites (DLA) tumor cell line. Interestingly, the acetone extract of rhizome showed much lower LC_{50} value (14.27 µg/ mL) for the DLA cell line compared to that of normal rat spleen cells (1189.23 µg/mL), revealing its significant anticancer potential. The molecular docking analysis showed β-sitosterol, present in the rhizome, as a promising lead molecule for the development of cytochrome P450 1 A1 inhibitors, which may provide potential anticancer agents.

Keywords: Cancer, *Keerikkizhangu*, Noncommunicable disease (NCD), Tumor, Wight's Twisted Arum

Cancer is a deadly disease characterized by abnormal and uncontrolled cell growth which destroy body tissues. It is one of the major non communicable diseases affecting 8.1 million patients causing 16% deaths worldwide next only to cardiovascular diseases (31%). Asia is reported to be leading both in cancer incidence (57.3%) as well as in cancer mortality (48.4%). In the Age-Standardized Rate (ASR) of cancer incidence and mortality globally (24 world areas) for all cancers combined, India ranks 18th in cancer incidence with ASR 279.8 and the least (24th) in mortality with ASR 123[1-3]. The United States is projected to record 1.81 million new cancer cases and 0.61 million deaths in 2020; approximately, 4950 new cases and 1660 deaths each day[2]. Even though early detection of various cancers helps in proper treatment and curing, the side effects associated with the treatment procedures such as radiation therapy, chemotherapy, etc. make cancer the most threatening disease. Treatment methods such as chemotherapy and radiotherapy result in severe side effects on patients, ranging from nausea, allergic reactions, decreased immune response to bleeding and toxicity. Patients also endure from the chance of metastasis, with future complications like formation of tumours in other parts of the body[4].

Plants provide a hope as a dependable resource of anticancer compounds without any side effects unlike synthetic drugs[5-9]. For mankind, there is an urgent need to explore newer resources to develop therapeuticsto overcome such a dreaded disease. Hence, researchers are focussed on developing such novel anticancer compounds from medicinal plant resources[10-12].

Anaphyllum wightii Schott., belonging to Araceae (Arum family), commonly called Wight's Twisted Arum, and locally in Kerala as *Keerikkizhangu*, is an ethnomedicinal plant used by tribal communities of Kerala such as Kani, Kadars, Madhuvars, etc.[13]. It is a tall herbaceous plant belonging to the family Araceae which is an endemic and threatened species of South India[14,15]. The plant is having a rhizomatous stem, pinnately compound leaves, with two major variations, either broad or narrow and spadix inflorescence with a characteristically twisted spathe. Tribal people use the rhizome of this plant as an antidote against snake bite and also as food[13,16]. The rhizome is reported to have various pharmacological properties such as antibacterial, anthelmintic, antioxidant, hepatoprotective, anti-inflammatory,

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and antidiabetic activities which support the ethno-botanical evidences[13].

β-Sitosterol, a potential anticancer compound[17], already considered as a standard reference for herbal drug comounds[18], has been reported from other Araceae members[19]. However, there are no previous reports available on the anticancer potential of *Anaphyllum wightii*. Hence, in the present study, we assessed the *in vitro* cytotoxic effects of this plant on Dalton's lymphoma ascites (DLA) cell lines and thereby evaluate the anticancer property of the plant. This study also involved the molecular docking analysis of β-sitosterol against target proteins involved in cancer development.

Materials and Methods

Plant material

Both broad and narrow-leaved varieties of *Anaphyllum wightii* were collected from Kallar, Kerala, identified by a taxonomic expert from the University of Kerala, and were planted in the garden of Dept. of Botany, University of Kerala. The leaves of both varieties and the rhizome of only the broad-leaved variety, which is commonly used in tribal medicine, were used for the *in vitro* cytotoxicity assays.

Soxhlet extraction

About 12 g of the powdered rhizome (of broad-leaved variety) and two leaf varieties were subjected to serial soxhlet extraction for about 6-8 h using 120 mL each of the solvents such as petroleum ether, chloroform, acetone, methanol and distilled water in the increasing order of polarity. Then methanolic extracts of the two leaf varieties and the methanolic, as well as acetone extracts of the rhizome, were selected for the *in vitro* cytotoxicity assay since, in case of the rhizome, acetone extract was more active than the methanolic extract. Only the acetone and methanolic extracts were selected for the study because these two were found to have higher antioxidant potential compared to other solvent extracts in our previous study (Unpublished data).

In vitro cytotoxicity assay

The short term *in vitro* cytotoxic effects of the rhizome and leaf extracts were studied using Dalton's lymphoma ascites (DLA) cell line by trypan blue exclusion method[20] using a hemocytometer. The tumor cells aspirated from the peritoneal cavity of tumor-bearing mice were washed thrice with phosphate-buffered saline (PBS) or normal saline. Viable cell suspension (1×10^6 cells in 0.1 mL) was added to the tubes with different concentrations of the extracts and later made up to 1 mL using PBS. The tube containing only cell suspension (without the extract) was used as the control. These assay mixtures were incubated for three hours at 37°C. Further, the cell suspension was mixed with 0.1 mL of 1% trypan blue, loaded on a hemocytometer after 2-3 min. Live cells do not absorb the blue colour of trypan blue but dead cells take up the colour of the dye. The number of stained and unstained cells were counted separately, and the percentage of cell death was calculated. The assay was done using normal rat spleen cells also.

HPTLC Fingerprinting

About 25 µL each of the sample extracts (100 µg/µL) and standards (1.0 mg/mL) were applied as bands of width 8 mm on silica gel 60 F_{254} pre-coated aluminium sheets through CAMAG microliter syringe using Automatic TLC Sampler 4 (ATS4). After sample application, the plate was introduced vertically in a CAMAG developing chamber (10×10 cm) pre-saturated with the mobile phase. A number of solvent systems were tried, and a system that gave the maximum resolution was selected as the solvent system for the extract. The optimum separations of constituents were achieved using toluene: ethyl acetate: formic acid (5: 3: 0.1) as the mobile phase. The developed chromatogram was air-dried to evaporate solvents from the plate, and the plate was kept in CAMAG Visualizer, and the images were captured under UV light at 254 and 366 nm[18].

Post chromatographic derivatization

The plate was derivatized using vanillin-sulphuric acid reagent, heated at 105°C by placing on CAMAG TLC plate heater till the colour of the bands appeared. Then the plate was visualized under white light, and the chromatograms were documented.

Molecular docking analysis

Molecular docking analysis of β-sitosterol was done using Autodock software against the target Cytochrome P450 1 A1. The docked conformation of the ligand-protein complex was visualized using the Discovery studio visualizer.

Statistical analysis

The values are expressed as mean ± SE. A value of $P < 0.05$ was considered significant.

Results and Discussion

The *in vitro* cytotoxicity assays can be considered as the preliminary screening methods to evaluate the anticancer potential of plant extracts. In the present study, the short term *in vitro* cytotoxic effects of acetone, as well as methanolic extracts of the rhizome and the methanolic extracts of the two leaf varieties, were done using Dalton's lymphoma ascites (DLA) cell line. Lymphoma cancer, a heterogeneous group of malignant disease with a wide spectrum of illnesses, comprises 70 different subtypes, and is observed most commonly in children, next to Leukemia[21]. Dalton's lymphoma is a tumor that originated in the thymus gland of a DBA/2 mouse at the National Cancer Institute, Bethesda, US, in 1947. Subsequently, an ascites form was developed by repeated intraperitoneal transplantation of tumors. It is highly invasive in nature, and kills the host in a concise period of life span[22].

The percentage of cytotoxicity was calculated by the trypan blue exclusion method. Trypan blue is a blue-coloured dye that can penetrate into dead cells and stain them, whereas living cells will remain unstained. Thus after treating the cell lines with the extracts, the exact number of dead and viable cells can be counted by using this dye[23]. The percentage of cell death effected by the four extracts at different concentrations are represented in Table 1.

Among the rhizome and leaf extracts selected for the assay, the acetone extract of rhizome showed the lowest LC_{50} value (14.27 µg/mL) for DLA cell line followed by the methanolic extract of rhizome (39.27 µg/mL), whereas these two extracts showed higher LC_{50} values for the normal rat spleen cells (1189.23 and 2468.55 µg/mL, respectively) as shown in Table 1. The LC_{50} value of well-known reported anticancer compound curcumin[24], was comparable to that shown by the acetone extract of rhizome of *A. wightii*. The methanolic extracts of both the narrow and broad leaves showed higher LC_{50} values for the DLA cell line (910.23 and 2468.55 µg/mL, respectively) compared to that of rhizome extracts.

Lower the LC_{50} value, higher will be the cytotoxicity. A potential anticancer compound should exhibit a lower LC_{50} value for cancer cell lines and a comparatively higher LC_{50} value for the normal cells. Thus, the results of the trypan blue dye exclusion technique indicated that both the acetone as well as methanolic extracts of rhizome could inhibit the growth of DLA cells significantly in a concentration-dependent manner and hence may contain potential anticancer compounds.

β-Sitosterol is a well-known phytosterol commonly found in the family Araceae, and it is reported as one of the major bioactive compounds in the tubers of *Colocasia esculenta*[25], *Amorphophallus paeoniifolius*[26] and *Amorphophallus companulatus*[27]. Earlier studies reported that β-sitosterol has significant cytotoxic potential against the cancer cell lines HT-29 (colon cancer)[28] LNCaP (prostate cancer), MDA-MB-231 (breast cancer)[29], HL60 (Caucasian promyelocytic leukemia)[30], U937 (human leukemic cells), COLO320 (human colorectal cancer cells)[31], and MCA-102 (fibrosarcoma cells)[32]. However, this compound was not previously reported in this particular plant, and hence we did HPTLC analysis of the rhizome and leaf extracts using the standard β-sitosterol which confirmed the presence (Fig. 1).

Since β-sitosterol has been reported as an anticancer agent, molecular docking of the same was done against the target protein cytochrome P450 1 A1 (CYP 1 A1). CYP 1 A1 is one of the cytochrome P450 enzymes involved in the activation of carcinogenic compounds[33]. It can convert polycyclic aromatic hydrocarbons to carcinogenic compounds. Hence, the inhibitors of this enzyme may be useful in prevention or inhibition of various cancers. β-sitosterol showed significant interaction with the

Table 1 — *In vitro* cytotoxicity of rhizome, broad and narrow leaves

Sample concentration (µg/mL)	Percentage of cell death (%)							
	Normal cell line (Rat spleen cells)				Dalton's lymphoma ascites cell line			
	Rhizome		Broad leaves	Narrow leaves	Rhizome		Broad leaves	Narrow leaves
	Acetone	Methanol			Acetone	Methanol		
10	0±0.00	0±0.00	0±0.00	0±0.00	38.7±0.75	26.5±0.00	0±0.00	0±0.00
20	0±0.00	0±0.00	0±0.00	0±0.00	53.0±0.23	46.9±0.95	0±0.00	0±0.00
50	0±0.00	0±0.00	6±0.00	4±0.00	87.7±1.14	58.3±0.75	3±0.00	0±0.00
100	2±0.00	0±0.00	12±0.00	5±0.00	93.8±0.90	83.6±0.83	7±0.52	0±0.00
200	8±0.00	4±0.00	20±0.83	9±0.00	100±0.00	91.8±0.61	10±0.00	4±0.32
LC_{50} values (µg/mL)	1189.23	2468.55	467.53	1064.66	14.27	39.27	910.23	2468.55

[Values are Mean ± Standard error; n = 3]

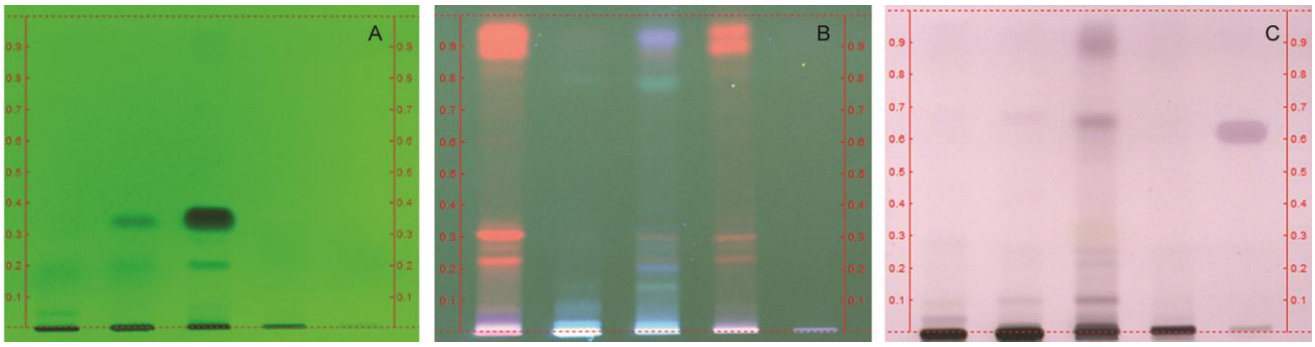

Fig. 1 — HPTLC profile of rhizome and leaf extracts of *Anaphyllum wightii* viewed in (A) UV short; (B) UV long; and (C) after derivatization using vanillin-sulphuric acid viewed in visible light.[Solvent system: toluene:ethylacetate:formic acid @ (5:3:0.1); Volume applied: Track 1, Narrow leaf (methanolic extract) 25 µL; Track 2, Rhizome (methanolic extract) 25 µL; Track 3, Rhizome (acetone extract) 25 µL; Track 4, Broad leaf (methanolic extract) 25 µL; and Track 5, β-sitosterol (25 µL)]

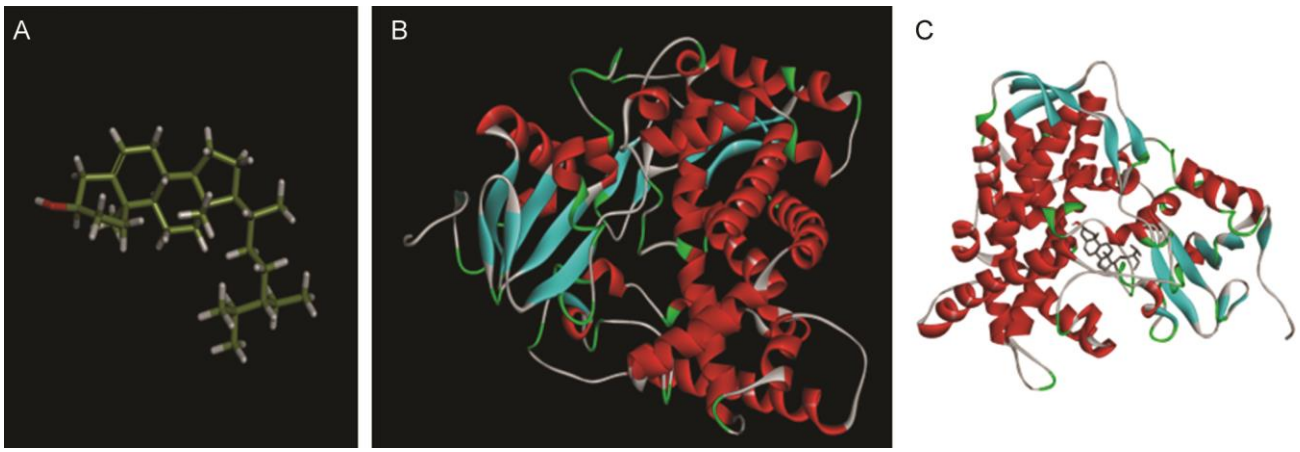

Fig. 2 — Three-dimensional structure of the ligand (A) β-sitosterol; (B) protein CYP 1 A1; and (C) the ligand-protein complex

target protein cytochrome P450 1 A1 with the binding energy of −10.69 KCal/mol. This lower binding energy indicates that the ligand (β-sitosterol) fits into the active site of the protein CYP 1 A1 more strongly. The docked conformation of the ligand-protein complex is represented in Fig. 2.

The docking analysis revealed that β-sitosterol might be a promising lead structure for the development of cytochrome P450 1 A1 inhibitors since it can interact significantly with the protein and thus may act as potential anticancer agents.

Conclusion

In the present study, we have determined the *in vitro* cytotoxic effects of *Anaphyllum wightii* on Dalton's lymphoma ascites (DLA) tumor cell line and also assessed the potential of its constituent βa-sitosterol to serve as a lead molecule for drug development. The rhizome extract of *A. wightii* showed significant *in vitro* cytotoxicity against the DLA cell line which suggests that it may contain compounds with anticancer potential which was supported by docking studies. The compound β-sitosterol present in the plant can act as a lead compound for the development of CYP 1 A1 inhibitors, and hence it may be useful in cancer therapy.

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Conflict of Interest

Authors declare no conflict of interests.

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